# STUDIES ON SHELL FORMATION. IV. THE RESPIRATORY METABOLISM OF THE OYSTER MANTLE[1, 2]

LOUISE H. JODREY[3] AND KARL M. WILBUR

*Department of Zoology, Duke University, Durham, N. C.*

One of the major problems in the study of shell formation is the relation of respiratory pathways to the deposition of the $CaCO_3$ and the organic matrix. In addition to the participation of respiration in the synthesis of organic constituents, respiration may also be involved in the transport of organic and inorganic substances through the cells of the mantle tissue to the site of shell deposition. Further, $CO_2$ from respiratory intermediates may provide a source of shell carbonate. Since the respiratory pathways of the shell-forming tissues of mollusks are unknown, the first requirement, and one of the objectives of this series of studies, is their identification. The present study concerns oxidative phases, and includes assays of oxidative enzymes and the effects of various substrates and respiratory inhibitors on oxygen consumption. Decarboxylation reactions will be the subject of another report.

It has long been recognized that the mantle is intimately concerned in processes of shell formation. Recently Hirata (1953) demonstrated that the isolated mantle of the oyster has the capacity to deposit both the organic and inorganic portions of the shell, and that this capacity is maintained for a relatively long period after isolation. The fact that the mantle has within itself the mechanisms of shell formation means that *initial* studies of the respiratory reactions can be confined to this structure. Once the respiratory activities of the mantle are established, one will then be in a position to relate these to the deposition of shell substances.

## Methods

The earlier portions of the respiratory studies were carried out on oysters from East Bay, Pensacola, Florida, during the period February to May, 1952, and continued on oysters from Core Creek, Beaufort, N. C., during July to November, 1952. Specimens of *Crassostrea virginica* (formerly *Ostrea virginica*), 8.0 cm. to 11.5 cm. in length (average 9.6 cm.), were collected in deep water and maintained

[1] Supported by a grant from the Office of Naval Research under Contract No. N7onr-45505 with Duke University.

[2] We are deeply indebted to Dr. P. A. Butler, Shellfishery Laboratory, Pensacola, Fla., Dr. W. A. Chipman and Mr. G. B. Talbot, U. S. Fishery Laboratory, Beaufort, N. C., and Dr. George Brecher of the National Institute of Health for placing the facilities of their laboratories at our disposal. We wish to express our appreciation to Dr. Marvin Schneiderman of the National Institute of Health who has been most generous of his time in carrying out certain statistical analyses and in advising concerning statistical procedures. The assays of oxaloacetic decarboxylase and certain of the fumarase assays were carried out by Mr. Samuel P. Maroney, Jr. and Mr. Peter Heneage.

[3] Present address: Department of Physiology and Pharmacology, University of Nebraska College of Medicine, Omaha.

below low tide level at the laboratory. Temperature and salinity ranges were as follows: Beaufort: temperature, 17.2°–31.0°; salinity 24.6–35.2; Pensacola: temperature, 10.0°–27.5°; salinity, 18.8–27.0. Enzyme assays were performed on Beaufort oysters from Core Creek and North River during May to July, 1953. Since the salinity near the laboratory where the oysters were maintained was higher than at the collecting sites, all oysters were held from 10 to 14 days before being used. During this period the temperature range was 20°–30° and the salinity range was 27.6–34.1.

*Respiration measurements*

Immediately after taking the specimens from the water the mantles were removed; cut into strips; drained for 10 seconds with one end touching filter paper; and weighed on a torsion balance. Respiratory measurements were carried out using Warburg's direct method on single strips of mantle weighing 150–220 mg. in 5-ml. flasks with sea water as the medium. The total fluid volume including tissue was 1.1 ml. The mantle is sufficiently thin that diffusion of oxygen is not limiting. Flasks were gassed with $O_2$ bubbled through sea water. All measurements were made at 25.0° except where noted. The pH of the medium was routinely measured prior to and frequently following runs. In the majority of cases the pH was not altered appreciably during the course of the respiratory measurements even with shaking for periods of 5–7 hours duration in the presence of alkali. Occasionally, however, there was a marked increase in the alkalinity from an initial pH of about 8 to 9.1–9.5. In a few cases a change of several tenths of a pH unit in the acid direction was also observed.

All substrates and inhibitors were made up in sea water. Adjustment of pH was made with HCl and NaOH. In studying the effect of a compound on respiration, $O_2$ consumption was measured during a 2- to 3-hour control period prior to the addition of the experimental compound from the side arm. After addition, measurements were continued for 3 to 4 hours. Oxygen consumption remained constant throughout the seven-hour period of these measurements but decreased slightly during the next few hours. The procedure recommended by Robbie (1948) was followed in using NaCN.

*Enzyme assay methods*

*Aconitase.* Spectrophotometric method of Racker (1950) using supernates from fresh homogenates. Homogenates were prepared by grinding a pooled sample of mantle tissue in a mortar followed by homogenization in 0.1 *M* K phosphate buffer, pH 7.4, in a glass homogenizer. The tissue was kept ice cold throughout. The supernatant following 10 minutes of light centrifugation was used for the enzyme assays. The increase in optical density at 240 mμ resulting from the conversion of citrate to unsaturated cis-aconitate was followed at intervals of 30 seconds. A unit of enzyme activity is the amount causing an increase in optical density of 0.001 log unit per minute at 22°–26°. Tissue activity is expressed as units of enzyme per mg. dry weight.

*Isocitric dehydrogenase.* Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract as prepared by Wenner *et al.* (1952), with slight

modifications, in glycylglycine buffer, pH 7.2. The reaction is started by the addition of isocitrate, and the increase in optical density at 340 mμ resulting from the reduction of TPN is read at 15-second intervals against a control cuvette containing all components except isocitrate. Enzyme activity is expressed in units per mg. of acetone powder, one unit being the amount of enzyme required to increase the optical density 0.01 log unit per minute at 22°–26°.

*Succinic dehydrogenase.* Spectrophotometric method of Cooperstein, Lazarow and Kurfess (1950). Assays were carried out on homogenates prepared with 10 volumes of 0.03 *M* Na phosphate buffer, pH 7.4, followed by further dilution, giving a final concentration of 1 : 30 in the cuvette. The increase in optical density at 550 mμ resulting from the reduction of cytochrome *c* was read at 30-second intervals. Measurements were made immediately following preparation of the homogenate and before clumping of particles became marked. Enzyme activity is expressed as the decrease in the logarithm of the molar concentration of oxidized cytochrome *c* per minute for a 1 : 150 tissue dilution.

*Cytochrome oxidase.* Spectrophotometric method of Cooperstein and Lazarow (1951). The tissue was prepared as for succinic dehydrogenase except that the final dilution was 1 : 300. The decrease in optical density at 550 mμ resulting from oxidation of cytochrome *c* was read at 30-second intervals. Enzyme activity was calculated as the decrease in the logarithm of the molar concentration of reduced cytochrome *c* per minute for a 1 : 100 tissue dilution.

*Fumarase.* Spectrophotometric method of Racker (1950) using a fresh homogenate and also an acetone powder extract as prepared by Wenner *et al.* (1952). The increase in optical density at 240 mμ resulting from the formation of the unsaturated fumarate from malate at pH 7.4 provides an index of enzyme activity. The calculation of unit enzyme activity is the same as for aconitase.

*Malic dehydrogenase.* Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract. The reaction was started by the addition of oxaloacetate, and the decrease in optical density at 340 mμ resulting from the oxidation of $DPNH_2$ was read at 15-second intervals. A pH of 7.2 was maintained with glycylglycine buffer. The calculation of enzyme activity is the same as for isocitric dehydrogenase.

*Oxaloacetic decarboxylase.* The manometric method of Vennesland *et al.* (1947) was employed using fresh homogenates at pH 4.5. Corrections were made for spontaneous breakdown of the substrate. Activity is expressed as μl. $CO_2$ per mg. dry wt. per hr. as calculated from $CO_2$.

## Results

### *Endogenous respiration*

Before considering the individual enzymes of the mantle and the effect of respiratory substrates, attention will first be given to endogenous respiration including the respiration of the different portions of the mantle. The possibility of metabolic differences between mantle areas is suggested by structural differences, differences in calcium turnover rates (Jodrey, 1953), and by differences in the rate of calcium deposition between areas of the inner shell surface (Wilbur and Jodrey, 1952). A difference in $CO_2$ content of mantle areas of the clam *Venus mercenaria* has also been reported (Dugal, 1939).

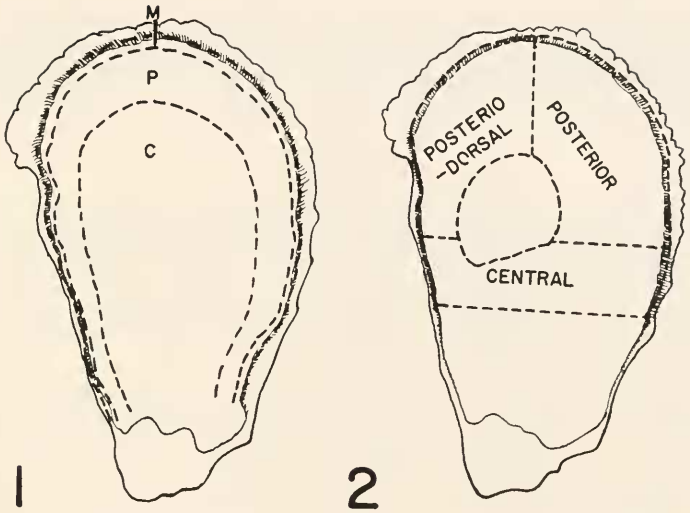


FIGURE 1. Mantle marked off to show zones used for respiratory measurements on Florida oysters. M, marginal zone; P, pallial zone; C, central zone.

FIGURE 2. Mantle marked off to show regions used for respiratory measurements.

For experimental purposes the oyster mantle may be considered to consist of three concentric zones (Fig. 1): a thin outer folded margin (M); an area attached to the shell and here termed the pallial zone (P); and a larger, thinner central zone (C). Measurements of endogenous respiration were carried out on the three zones (Table 1). The pallial zone exhibited the highest rate and the central the lowest. Differences between zones were significant at the 1% level. In subsequent experiments mantle regions (Fig. 2) including all three zones were utilized for respiratory measurements. The endogenous respiration in North Carolina oysters was the same for the three mantle regions. In Florida oysters the three regions appeared to exhibit differences in respiration, but the data, which were obtained under conditions of temperature and season quite different from the North Carolina oysters, are not sufficiently extensive to warrant discussion. In order that any such difference would not figure in the results, experimental and control tissues were always taken from corresponding mantle regions.

TABLE I

*Endogenous respiration of mantle zones*

| Mantle zone | $\mu$l. $O_2$/mg. wet wt./hr. |
|---|---|
| Marginal | $0.13 \pm 0.03$ |
| Pallial | $0.15 \pm 0.03$ |
| Central | $0.12 \pm 0.02$ |

Mantles were divided into three zones as shown in Figure 1 and the oxygen consumption of the parts measured simultaneously. Florida oysters. Environmental temperatures, 10°–14° C. Measurements were carried out at constant temperatures between 18° and 21°. Salinity 24–25 parts per thousand. Figures show means and standard deviations, $\sqrt{\frac{\Sigma d^2}{n-1}}$.

A further aspect of endogenous respiration deserves mention, namely, the effect of short-term starvation of isolated mantles. Right mantles with the attached shell were isolated (Hirata, 1953) and kept for periods of one to 7 days in running sea water. The endogenous respiration of the three regions shown in Figure 2

TABLE II

*Respiratory enzymes in mantle*

| Enzyme | Mantle | Mammalian tissue | | Reaction |
|---|---|---|---|---|
| Aconitase | none | mouse liver<br>rat kidney<br>rat liver | 33<br>50<br>25* | $\text{citrate} \overset{\pm H_2O}{\rightleftharpoons} \text{aconitate} \overset{\pm H_2O}{\rightleftharpoons} \text{isocitrate}$ |
| Isocitric dehydrogenase | 0.36 | mouse liver<br>rat kidney<br>rat liver | 10.8<br>66<br>22* | $\text{isocitrate} + \text{TPN} \overset{+2H}{\rightleftharpoons} \text{oxalosuccinate} + \text{TPNH}_2$ |
| Succinic dehydrogenase | 0.03 | rat liver | 2.96 | $\text{succinate} + \text{ferricytochrome c} \overset{\pm 2H}{\rightleftharpoons} \text{fumarate} + \text{ferrocytochrome c}$ |
| Cytochrome oxidase | 0.61 | rat liver<br>rat kidney | 11.85<br>23.16 | $\text{ferrocytochrome c} + 1/2\ O_2 \longrightarrow \text{ferricytochrome c} + H_2O$ |
| Fumarase | 2.0±<br>0.3 | mouse liver<br>rat kidney<br>rat liver | 132<br>62<br>77* | $\text{fumarate} \overset{\pm H_2O}{\rightleftharpoons} \text{malate}$ |
| Malic dehydrogenase | 3.3 | mouse liver<br>rat kidney<br>rat liver | 256<br>173<br>87* | $\text{malate} + \text{DPN} \overset{+2H}{\rightleftharpoons} \text{oxaloacetate} + \text{DPNH}_2$ |
| Oxaloacetic decarboxylase | 592±<br>187 | mouse liver | 3.2 | $\text{oxaloacetate} \overset{\pm CO_2}{\rightleftharpoons} \text{pyruvate}$ |

*Aconitase:* supernatant from light centrifugation of 1:20 homogenate in 0.1 *M* K phosphate buffer, pH 7.4, 0.1 ml.; 0.05 *M* phosphate buffer, pH 7.4, 2.4 ml.; 0.03 *M* sodium citrate, 0.5 ml. *Isocitric dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; $6.6 \times 10^{-4}$ *M* TPN, 0.45 ml.; 0.05 *M* $MnCl_2$, 0.05 ml.; 1:15 acetone powder extract, 1.0 ml.; 0.01 *M* sodium isocitrate, 0.5 ml. *Succinic dehydrogenase:* 0.1 *M* sodium succinate in 0.1 *M* Na phosphate buffer, pH 7.4, 0.5 ml.; 0.03 *M* NaCN in 0.17 *M* phosphate buffer, pH 7.4, 0.2 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 1.0 ml.; 100 mg.% cytochrome in 0.17 *M* phosphate buffer, pH 7.4, 1.0 ml.; water to 3.0 ml. *Cytochrome oxidase:* 60 mg.% cytochrome in 0.03 *M* phosphate buffer, pH 7.4, 3.0 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 0.1 ml. *Fumarase:* 0.5 ml. of 1:40 homogenate in double distilled water; 0.1 *M* potassium malate, 1.5 ml.; 0.1 *M* K phosphate buffer, pH 7.4, 0.75 ml.; double distilled water, 0.25 ml. *Malic dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; $1.7 \times 10^{-3}$ *M* $DPNH_2$, 0.2 ml.; 1:15 acetone powder extract, 0.1 ml.; 0.01 *M* sodium oxaloacetate 1.0 ml., water to 3.0 ml. *Oxaloacetic decarboxylase:* 0.1 *M* acetate buffer, pH 4.7, 0.25 ml.; $6.6 \times 10^{-4}$ *M* TPN, 0.05 ml.; $10^{-2}$ *M* $MnCl_2$, 0.1 ml.; 1:10 homogenate in 0.1 *M* acetate buffer, 0.5 ml.; $7.6 \times 10^{-2}$ *M* oxaloacetate, 0.1 ml. (1 mg. per flask).

Figures for mantle enzymes other than fumarase and oxaloacetate represent pooled samples of 9 or more mantles. Starred figures on mammalian tissues obtained in the present study. Other data on mammalian tissues from Wenner *et al.* (1952) except succinic dehydrogenase and cytochrome oxidase which are from Cooperstein *et al.* (1950) and Cooperstein and Lazarow (1951), respectively.

was then measured. Surprisingly, the respiration per mg. wet weight did not change significantly with the period of isolation, nor was it different from that of mantles taken directly from unstarved oysters. Such metabolic stability, especially in the absence of added substrate, is indeed remarkable.

*Enzyme assays*

Table II presents the results of the assays of mantle enzymes, together with values for the same enzymes in certain mouse and rat tissues for comparison. Isocitric, succinic, and malic dehydrogenases were present and confirm earlier unpublished experiments on succinic and malic dehydrogenases carried out in collaboration with Dr. Henry Kritzler and Mr. Arthur A. Hirata using the Thunberg technic. Fumarase was found in fresh homogenates but not in acetone preparations. Assays of freshly prepared homogenates gave no indication of aconitase activity.

Close comparisons between the *in vivo* activity of the mantle and mammalian enzymes (Table II) are not justified even though the same assay methods were used for both. Under the conditions of assay, however, tricarboxylic cycle enzymes in mantle have a considerably lower activity than in the mammalian tissues.

TABLE III

*Effect of substrates on mantle respiration*

| Substrate | 0.005 *M* | 0.01 *M* | 0.025 *M* | 0.05 *M* |
|---|---|---|---|---|
| Isocitrate | —— | 26±8 | —— | —— |
| Citrate | −1±5 | 2±4 | —— | —— |
| Succinate | 10±5 | 15±9 | 18±8 | 26±3 |
| Malate | 13±5 | 20±5 | 16±7 | —— |
| Oxaloacetate | 13±8 | —— | 20±7 | 22±5 |
| Pyruvate | 8±10 | 3±7 | —— | —— |

Figures show mean percentage change in oxygen consumption and standard deviation following the addition of the substrates to mantle.

On the other hand, this is not the case for oxaloacetic decarboxylase. This enzyme had an activity in the mantle which is two orders of magnitude higher than that of mouse liver. This finding may be of special significance for the mantle tissue which is concerned with processes of carbonate deposition. (See paper by Wilbur and Jodrey which follows the present paper.)

*Substrate effects*

Several substrates of the tricarboxylic acid cycle have been added to pieces of mantle (Fig. 2) and their effects on oxygen consumption measured (Table III). Isocitrate, succinate, malate, and oxaloacetate stimulated respiration, the effect becoming greater as the concentration was increased above 0.005 *M*. The maximal stimulation amounted to 20–26%. Oxidative enzymes for these four substrates were demonstrated in the previous section (Table II). Citrate was without effect, and this result is correlated with the failure to demonstrate aconitase. In the absence of aconitase citrate would not be converted to isocitrate which can

be oxidized by the mantle. Pyruvate also did not alter oxygen consumption appreciably. The possible "sparking" action of acetate was not studied.

The question of penetration of the substrate arises in cases of the negative results with pyruvate and especially with citrate which would form calcium and magnesium salts in the sea water medium.[4] Ronkin (1950) found that the effect of respiratory inhibitors on the gill of the mussel *Mytilus* was increased by decreasing the pH of the medium, presumably from increased penetration of the undissociated molecule. The effect of pH was examined with succinate, pyruvate, and citrate (Table IV). Pyruvate and citrate exhibited no significant effect on the mean respiration over the range pH 6.0–8.4. However, certain mantles were stimulated and others were inhibited as indicated by the standard deviation values. The stimulating action of succinate remained constant between pH 6.6 and 8.4 and was abolished below pH 6.6, although the level of endogenous respiration was

Table IV

*Effect of pH on mantle respiration*

| Substrate | pH | | | | |
|---|---|---|---|---|---|
| | 6.0–6.2 | 6.3–6.5 | 6.6–7.4 | 7.5–7.9 | 8.0–8.4 |
| Succinate, 0.05 *M* | 2.1±11(6) | 0.1±6.8(4) | 24±11(6) | 26±7.5(12) | 27±9.2(7) |
| Pyruvate, 0.01 *M* | −6.2±5.7(2) | —— | −2.2±11(2) | —— | 2.5±10(2) |
| Citrate, 0.01 *M* | 2.1±6.1(11) | —— | 1.5±12(5) | —— | 1.6±20(5) |
| Sea water | 0.15 | 0.20 | 0.21 | 0.18 | 0.17 |

Oxygen consumption was first measured for 2–3 hours in sea water, the pH of which had been altered by the addition of HCl or NaOH. Substrates made up in sea water of the same pH were then tipped in and the measurements of oxygen consumption were continued for several hours. The pH was again measured at the end of the experiment. The mean percentage change and standard deviation of the respiratory rate resulting from the addition of substrate are given. $O_2$ consumption in $\mu$l/mg. wet wt./hr. Figures in parenthesis show the number of cases. At pH 6 the calculated dissociation is 74% for succinate and 80% for citrate. Data were not available for a calculation for pyruvate.

maintained very nearly at the normal level to a pH of 6.0–6.2. This latter result poses an interesting problem in that one might expect endogenous respiration to be depressed to a greater degree if succinate is in the respiratory cycle.

We have mentioned that isolated mantles can be maintained in good condition for several days without added substrate. Nine such mantles isolated for 2 to 7 days have been compared with 7 freshly dissected mantles with respect to their respiration in the presence of 0.05 *M* succinate. The mean respiration ($\mu$l $O_2$/mg. wet wt./hr.) was 25 and 23, respectively ($P < 50$), demonstrating that both groups respond similarly. This result is not especially surprising in view of the unchanged endogenous respiration.

*Respiratory inhibitors*

In the two previous sections the presence of succinic dehydrogenase and cytochrome oxidase in mantle has been demonstrated. One can therefore expect that compounds known to inhibit the succinoxidase system will affect mantle respira-

[4] However, isocitrate, which would be expected to behave similarly, apparently penetrates.

tion. The action of such enzyme inhibitors and the copper-complexing agent diethyldithiocarbamate has been examined and the results are presented in the following sections.

*Sodium cyanide.* Endogenous $O_2$ consumption of mantle in the presence of $2 \times 10^{-4}$ *M* NaCN averaged only 7% of the control value without cyanide. The inhibitory action of cyanide was completely reversible after an exposure of 1¼ hours (Fig. 3) but only partially reversible after longer periods.

Methylene blue, which typically reverses at least a portion of cyanide inhibition (Thunberg, 1918), increased the oxygen consumption in a saturated solution in sea water from the cyanide level of 7% to a new level of 10%, and thus was

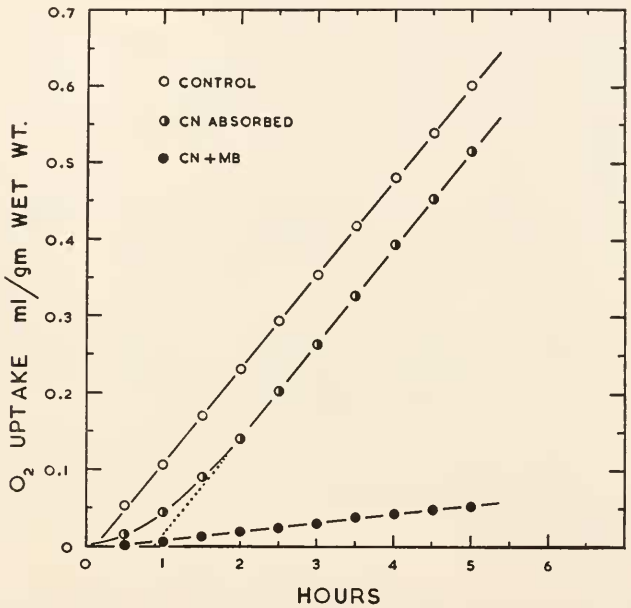


FIGURE 3. The effect of cyanide on mantle respiration. Lower curve: mantle in $2 \times 10^{-4}$ NaCN; saturated solution of methylene blue (final concentration $1.4 \times 10^{-5}$ *M*) tipped in at one hr. The change from 7% of control respiration to 10% after the addition of methylene blue is not perceptible on the graph. NaCN present in center well to prevent absorption of cyanide by KOH. Middle curve: mantle in $2 \times 10^{-4}$ *M* NaCN for 1¼ hr. in Warburg flask prior to 0 time on graph. At 0 time KOH without cyanide was added to center well of flask to absorb HCN. Oxygen uptake returned to the control value as cyanide concentration decreased.

without appreciable effect. This result is not due to failure of penetration since uptake of methylene blue by the cells of the whole mantle can be clearly seen under the microscope. Methylene blue when added to normal mantles neither altered the endogenous respiration nor the increased oxygen consumption produced by succinate. The absence of an increased succinate effect in the presence of a saturated solution of methylene blue ($6.3 \times 10^{-5}$ *M*) was shown in 12 experiments in which the average respiratory increase amounted to 21.2% as compared with 20.5% for 0.005 *M* succinate alone. From this result it appears that the cytochrome system is not limiting in succinate oxidation.

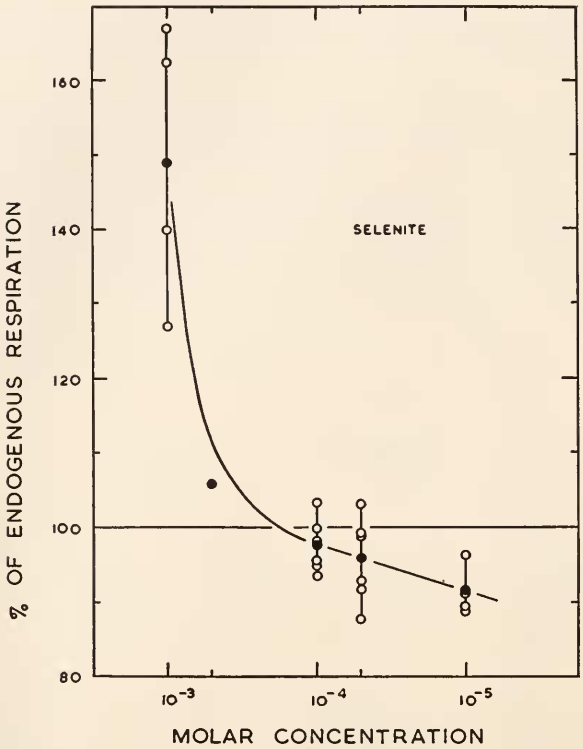


FIGURE 4. The effect of sodium selenite on mantle respiration. Solid circles show mean values. Open circles give values for individual experiments. Endogenous $O_2$ uptake was measured for 1–2 hrs.; selenite was then tipped in and measurements were continued. Note that the abscissa is in log units.

TABLE V

*Effects of diethyldithiocarbamate and ethyl carbamate on mantle respiration*

| | Mean per cent inhibition | | |
|---|---|---|---|
| | $10^{-4}$ *M* | $10^{-3}$ *M* | $10^{-2}$ *M* |
| Diethyldithiocarbamate | 8 | 32* | 13 |
| | 16 | 27 | 14 |
| | 10 | 22 | 7 |
| | 21 | 26 | 0 |
| Ethyl carbamate | 4 | 3 | 5 |
| | −1 | 1.5 | 4 |

The figures represent individual experiments and show the percentage change in $O_2$ consumption. North Carolina oysters.

* Maximum inhibition of 57% was found in Florida oysters.

*Sodium selenite.* Selenite has been studied in view of its inhibition of succinic dehydrogenase (Stotz and Hastings, 1937). The results of selenite on endogenous respiration are summarized in Figure 4 which demonstrates a dual effect of this compound. A slight inhibition occurred with $10^{-5}$ *M* with progressively less inhibition as the concentration was increased; and at $10^{-3}$ *M* a mean increase in respiration of 49% was observed. Measurements using selenite alone in sea water showed that the autoxidation of this compound would not account for the observed increase in oxygen consumption.

Selenite in a concentration of $10^{-5}$ *M*, which depressed endogenous respiration slightly, had a variable effect on respiratory increase from added succinate. In some cases the selenite reduced or abolished the succinate effect; in others it did not.

*Sodium malonate.* Malonate characteristically inhibits succinic dehydrogenase competitively. Mantle tissue treated with 0.025 *M* malonate prior to the addition of 0.005 succinate showed a 38% inhibition of the succinate effect which was significant at the 5% level. Malonate (0.025 *M*) was without effect on endogenous respiration. Cleland (1950) found only slight inhibition of the succinoxidase of unfertilized oyster eggs by malonate.

*Sodium diethyldithiocarbamate.* Endogenous respiration was inhibited by diethyldithiocarbamate, the maximum inhibition occurring at $10^{-3}$ *M* concentration with less inhibition at lower and higher concentrations (Table V). Ethyl carbamate (urethane), an inhibitor of dehydrogenase activity (Keilin, 1925; Stotz and Hastings, 1937), did not affect endogenous respiration in the same concentrations used for diethyldithiocarbamate. Assuming penetration of ethyl carbamate, the difference in action of the ethyl and diethyldithio compounds points to specificity of the sulfur-containing compound and, further, suggests that a copper catalyst may be an important part of the respiratory system.

## Discussion

Our objective in this series of experiments has been to establish a background of respiratory data for an understanding of sources of energy and carbonate for shell formation. In so doing we have first described the general respiratory picture of various parts of the mantle of Florida oysters and have found that the pallial zone immediately central to the folded outer margin has a slightly but significantly higher endogenous respiration than either the marginal or central area. It is this pallial zone of the shell which consistently showed the most rapid deposition of calcium in North Carolina oysters previously studied (Wilbur and Jodrey, 1952). As interesting as this apparent correlation may be, the comparison of two sets of data taken at different times and on oysters from different geographical locations must be viewed with caution.

While all steps of the tricarboxylic cycle have not been examined in mantle, enzyme assays and $O_2$ consumption measurements using several substrates have demonstrated that the major portion of the cycle is present. One apparent difference is the absence of aconitase which catalyzes the conversion of citrate to cis-aconitate and isocitrate. The cycle could operate, however, with isocitrate rather than citrate as the first compound. The isocitrate could then be converted to oxalosuccinate through the action of isocitric dehydrogenase. In homogenates of

the egg of the oyster *Ostrea commercialis* citrate increases oxygen consumption (Cleland, 1951), indicating that in this material citrate is metabolized. While the precursors of the tricarboxylic acid in mantle are not known, pyruvate may well be involved even though its action on respiration could not be clearly demonstrated using strips of mantle. Cleland (1950, 1951) has found that glycolysis and pyruvate occur in the egg of the oyster and the tricarboxylic acid cycle appears to be present for the oxidation of the pyruvate.

The tricarboxylic acid cycle in addition to providing sources of energy would generate $CO_2$ which might furnish shell carbonate. The respiratory processes described may be expected to apply to other functions of the mantle as well: ciliary movement; mucus formation and secretion; electrolyte regulation; and so on. A direct attack on the relation of respiration to shell formation can be made by measuring the effect of respiratory substrates and inhibitors and combinations of these on shell deposition. (See following paper by Wilbur and Jodrey.)

The very nearly complete blockage of respiration by $2 \times 10^{-4}$ $M$ cyanide points to heavy metal catalysis. However, methylene blue had a negligible effect in reversing the inhibition, suggesting that the cytochrome system may not play a major role in oxidation in this tissue, and, further, that the system concerned may have a redox potential higher than that of methylene blue. A copper catalyst may be involved as indicated by the inhibition of respiration by diethyldithiocarbamate. This can scarcely account for all the oxygen consumption, however, since the maximum inhibition observed was 57%. Maximum inhibition of $O_2$ consumption by sodium diethyldithiocarbamate occurred at an intermediate concentration of the inhibitor, respiration being less depressed at higher and lower concentrations. Apparently the progressive binding of copper causes the oxygen consumption to decrease to a minimum with a second opposing reaction occurring either as a result of still further decrease in copper concentration or as another effect of the inhibitor. Selenite also exhibited a dual effect on endogenous respiration. With this compound the inhibition at low concentrations was slight and at higher concentration ($10^{-3}$ $M$) there was a stimulation of endogenous respiration. The decrease could be attributed to succinate inhibition, although malonate was without effect on endogenous respiration. Whether selenite at higher concentrations acts in a manner similar to dinitrophenol which also stimulates oxygen consumption in mantle (Maroney and Wilbur, unpublished results) remains to be studied.

Two pieces of evidence in addition to the methylene blue and the diethyldithiocarbamate findings raise the question as to the quantitative importance of the tricarboxylic acid cycle in mantle respiration. Lowering the pH of the medium below 6.6 completely prevented the increased respiration which normally occurs with added succinate without decreasing respiration below the usual endogenous level. Also, malonate left the endogenous respiration unaltered. Both procedures would be expected to decrease $O_2$ consumption by inhibiting succinate oxidation.

The stability of the oxidative mechanisms of the oyster mantle is noteworthy. The endogenous respiration remained relatively constant in mantles isolated for several days in sea water without added substrate, and these mantles responded to added succinate in the same manner as freshly dissected mantles. The endogenous respiration was also but little changed by altering the pH of the sea water medium from 8.4 to 6.0. Such constancy of respiratory processes would seem to indicate a highly developed control of the intracellular environment. The experi-

mental variations just mentioned may have their counterparts in conditions encountered by the animals such as periods without feeding and variation in the pH of the mantle environment when bathed by sea water as compared with periods when the valves are closed, although in the normal environmental situation the extremes of pH studied here are probably not reached (Dugal, 1939).

## Summary

1. The oyster mantle has been studied with respect to endogenous respiration, activity of various oxidative enzymes and response to intermediates of the tricarboxylic acid cycle, and respiratory inhibitors.

2. The endogenous respiration has been found to be similar for different mantle regions, though small differences were present. Endogenous respiration was not significantly altered by changes in the pH of the sea water medium between 6.0 and 8.4 or by isolation of mantles in sea water without added substrate for periods of a few days.

3. Isocitric, succinic and malic dehydrogenases, fumarase, and cytochrome oxidase were present in the mantle. Aconitase could not be demonstrated. Oxaloacetic decarboxylase was found in very high concentration. Its presence may be of significance with respect to the formation of shell carbonate.

4. Isocitrate, succinate, malate, and oxaloacetate stimulated respiration. Enzyme assays and the effects of added substrates indicated the presence of the major portion of the tricarboxylic acid cycle in mantle tissue, although all steps have not been examined. Citrate and pyruvate were without effect on respiration over the range pH 6.0–8.4.

5. Malonate inhibited succinate oxidation partially but did not alter endogenous respiration. Selenite reduced respiration slightly at $10^{-5}$ *M* but brought about a mean increase of 49% at $10^{-3}$ *M*. Cyanide, $2 \times 10^{-4}$ *M*, inhibited respiration almost completely. The inhibition was completely reversible after 1¼ hours but only partially reversible after longer periods. Methylene blue was ineffective in reversing cyanide inhibition.

6. Sodium diethyldithiocarbamate produced a maximum inhibition of 57% at $10^{-3}$ *M*. Higher and lower concentrations were less effective. Ethyl carbamate (urethane) was without effect at the concentrations employed with diethyldithiocarbamate. The results suggest a copper respiratory catalyst in mantle.

## LITERATURE CITED

Cleland, K. W., 1950. Intermediary metabolism of unfertilized oyster eggs. *Proc. Linnean Soc. N. S. Wales*, **75**: 296–319.

Cleland, K. W., 1951. The enzymatic architecture of the unfertilized oyster egg. *Austral. J. Exp. Biol. Med. Sci.*, **29**: 35–45.

Cooperstein, S. J., and A. Lazarow, 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.*, **189**: 665–670.

Cooperstein, S. J., A. Lazarow and N. J. Kurfess, 1950. A microspectrophotometric method for the determination of succinic dehydrogenase. *J. Biol. Chem.*, **186**: 129–139.

Dugal, L. -P., 1939. The use of calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. *J. Cell. Comp. Physiol.*, **13**: 235–251.

Hirata, A. A., 1953. Studies on shell formation. II. A mantle-shell preparation for in vitro studies. *Biol. Bull.*, **104**: 394–397.

JODREY, L. H., 1953. Studies on shell formation. III. Measurements of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$. *Biol. Bull.*, **104**: 398–407.

KEILIN, D., 1925. On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. *Proc. Roy. Soc. London, Ser. B*, **98**: 312–339.

MEHLER, A. H., A. KORNBERG, S. GRISOLIA AND S. OCHOA, 1948. The enzymatic mechanism of oxidation-reduction between malate or isocitrate and pyruvate. *J. Biol. Chem.*, **174**: 961–977.

RACKER, E., 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. *Biochim. Biophys. Acta*, **4**: 211–214.

ROBBIE, W. A., 1948. The use of cyanide in tissue respiration studies. *Methods in Medical Research*, **1**: 307–317. Editor-in-Chief: V. R. Potter.

RONKIN, R. R., 1950. The uptake of radioactive phosphate by the excised gill of the mussel *Mytilus edulis*. *J. Cell. Comp. Physiol.*, **35**: 241–260.

STOTZ, E., AND A. B. HASTINGS, 1937. The components of the succinate-fumarate-enzyme system. *J. Biol Chem.*, **118**: 479–498.

THUNBERG, T., 1918. Zur Kenntnis der Einwirkung tierischer Gewebe auf Methylenblau. *Skand. Arch. f. Physiol.*, **35**: 163–195.

VENNESLAND, B., E. A. EVANS AND K. I. ALTMAN, 1947. The effects of triphosphopyridine nucleotide and of adenosine triphosphate on pigeon liver oxalacetic carboxylase. *J. Biol. Chem.*, **171**: 675–686.

WENNER, C. E., M. A. SPIRTES AND S. WEINHOUSE, 1952. Metabolism of neoplastic tissues. II. A survey of enzymes of the citric acid cycle in transplanted tumors. *Cancer Res.*, **12**: 44–49.

WILBUR, K. M., AND L. H. JODREY, 1952. Studies on shell formation. I. Measurement of the rate of shell formation using $Ca^{45}$. *Biol. Bull.*, **103**: 269–276.

**AUTHORS' OMISSION.** Wilbur and Jodrey, Vol. 108, p. 359.

The possible relation of carbonic anhydrase to calcification of mollusc shell has been discussed at length by Stolkowski (Ann. Inst. Oceanogr., *26:* 1–113, 1951). This investigator and his collaborators have shown that benzene sulfamide inhibits shell regeneration in the snail *Helix aspersa* and calcification in the ciliate *Coleps hirtus* and the larvae of the sea urchins *Paracentrotus lividus* and *Arbacia aequituberculata;* but it is not certain that the effect of the inhibitor is limited to carbonic anhydrase.